Integration of whole-body [^{18}F]FDG PET/MRI with non-targeted metabolomics can provide new insights on tissue-specific insulin resistance in type 2 diabetes

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY NOTE

S1. MoDentify

MoDentify identifies modules of metabolites associated with a selected outcome, that was M-value in this case[1]. It gains statistical power from metabolomics cohorts originating from multiple tissues. In this case, we used MoDentify to compute partial correlations of pairs of metabolites for SAT and plasma, while regressing out the other metabolites. Partially correlated metabolites were then represented into a network which was used as the base to identify functional modules of metabolites for the unified set of tissues. A score maximization algorithm was used to "walk" the network and to identify modules that are significantly associated with M-value at FDR<0.1, while controlling for BMI, WHR, age and sex. MoDentify identified two modules that we visualized in Cytoscape (Supplementary Figure S5).

S2. Metabolic Profiling

Information about reagents, solvents, standards, reference and tuning standards, and stable isotopes internal standards are displayed in the Supplementary Note - 1.4 Metabolic profiling solvents.

1.1 Sample Preparation

Sample preparation of plasma was performed according to A et al.[2]. In detail, 900µL of extraction buffer (90/10 v/v methanol: water) including internal standards for both GC-MS and LC-MS (Supplementary Note - 1.4 Metabolic profiling solvents) were added to 100µL of serum. The samples were shaken at 30Hz for two minutes in a mixer mill and proteins were precipitated at +4°C on ice. Afterwards, the samples were centrifuged at +4°C, 14000 rpm, for 10 minutes. The supernatants, 200µL for both batches of the LC-MS analysis, and 200µL and 50µL, respectively for the two batches of the GCMS analysis, were transferred to micro vials and evaporated to dryness in a SpeedVac concentrator.

SAT samples were extracted as follows: 500µL of 2/1 (v/v) CHCl3: methanol (including D4-Cholic Acid) and, 100µL water (including 13C9-phenylalanine) and two tungsten beads were added to each sample (18-23mg). The samples were shaken at 30Hz for 3 minutes. The tungsten beads were removed, and the samples were left standing at room temperature for 30 minutes. Samples were centrifuged at 14000 rpm, +4°C for 3 minutes and 80µL of the aqueous phase was transferred to Eppendorf tubes. 320µL methanol (including D6-salicylic acid) was added to the Eppendorf tubes, whereupon remaining proteins were precipitated at -20°C for 1 hour. The samples were centrifuged for 10 minutes at 14000 rpm, +4°C and 50µL supernatant was taken out to GC vials and 200µL for LC-MS. Solvents were evaporated and the samples were stored at -80°C until analysis.

The remaining supernatants of each tissue were pooled and used to create tissue-specific quality control (QC) samples. Tandem mass spectrometry (MS/MS) analysis for LC-MS was performed on the QC samples for identification purposes. Samples were analysed in tissue-dependent batches according to a randomized run order on both GC-MS and LC-MS.

1.2 GC-MS

Derivatization and GC-MS analysis were performed as described previously[2]. SAT samples were derivatized in a final volume of 30µL rather than 90µL which was used for plasma[3].

1.2.1 Batch 1

1µL of the derivatized sample was injected in splitless mode by a CTC Combi Pal autosampler (CTC Analytics AG, Switzerland) into an Agilent 6890 gas chromatograph equipped with a 10m x 0.18mm fused silica capillary column with a chemically bonded 0.18 µm DB 5-MS UI

stationary phase (J&W Scientific). The injector temperature was 270°C, the purge flow rate was 20mL/min and the purge was turned on after 60 seconds. The gas flow rate through the column was 1mL/min, the column temperature was held at 70°C for 2 minutes, then increased by 40°C/min to 320°C, and held there for 2 minutes. The column effluent was introduced into the ion source of a Pegasus III time-of-flight mass spectrometer, GC/TOFMS (Leco Corp., St Joseph, MI, USA). The transfer line and the ion source temperatures were 250°C and 200°C, respectively. Ions were generated by a 70eV electron beam at an ionization current of 2.0mA, and 30 spectra/s were recorded in the mass range m/z 50-800. The acceleration voltage was turned on after a solvent delay of 150 seconds. The detector voltage was 1500-2000V.

1.2.2 Batch 2

0.5µL of the derivatized sample was injected in splitless mode by a L-PAL3 autosampler (CTC Analytics AG, Switzerland) into an Agilent 7890B gas chromatograph equipped with a 10m x 0.18mm fused silica capillary column with a chemically bonded 0.18µm Rxi-5 Sil MS stationary phase (Restek Corporation, U.S.). The injector temperature was 270°C, the purge flow rate was 20mL/min and the purge was turned on after 60 seconds. The gas flow rate through the column was 1mL/min, the column temperature was held at 70°C for 2 minutes, then increased by 40°C/min to 320°C, and held there for 2 minutes. The column effluent was introduced into the ion source of a Pegasus BT time-of-flight mass spectrometer, GC/TOFMS (Leco Corp., St Joseph, MI, USA). The transfer line and the ion source temperatures were 250°C and 200°C, respectively. Ions were generated by a 70eV electron beam at an ionization current of 2.0mA, and 30 spectra/s were recorded in the mass range m/z 50-800. The acceleration voltage was turned on after a solvent delay of 150 seconds. The detector voltage was 1800-2300V.

1.3 LC-MS

The LC-MS was performed identically for both batches. Before LC-MS analysis the sample was re-suspended in 10+10µL methanol and water. Batches from the samples were first analysed in positive mode, next the instrument was switched to negative mode and a second injection of the samples was performed.

The chromatographic separation was performed on an Agilent 1290 Infinity UHPLC-system (Agilent Technologies, Waldbronn, Germany). 2µL of each sample was injected into an Acquity UPLC HSS T3, 2.1 x 50mm, 1.8µm C18 column in combination with a 2.1mm x 5mm, 1.8µm VanGuard precolumn (Waters Corporation, Milford, MA, USA) held at 40°C. The gradient elution buffers were A (H2O, 0.1% formic acid) and B (75/25 acetonitrile:2-propanol, 0.1% formic acid), and the flow-rate was 0.5mL/min. The compounds were eluted with a linear gradient consisting of 0.1-10% B over 2 minutes, B was increased to 99% over 5 minutes and held at 99% for 2 minutes; B was decreased to 0.1% for 0.3 minutes and the flow-rate was increased to 0.8mL/min for 0.5 minutes; these conditions were held for 0.9 minutes, after which the flow-rate was reduced to 0.5mL/min for 0.1 minutes before the next injection.

The compounds were detected with an Agilent 6550 Q-TOF mass spectrometer equipped with a jet stream electrospray ion source operating in positive or negative ion mode. The settings were kept identical between the modes, with the exception of the capillary voltage. A reference interface was connected for accurate mass measurements; the reference ions purine (4µM) and HP-0921 (Hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine) (1µM) were infused directly into the MS at a flow rate of 0.05mL/min for internal calibration, and the monitored ions were purine m/z 121.05 and m/z 119.03632; HP-0921 m/z 922.0098 and m/z 966.000725 for positive and negative mode respectively. The gas temperature was set to 150°C, the drying gas flow to 16L/min and the nebulizer pressure 35psig. The sheath gas temp was set to 350°C and the sheath gas flow 11L/min. The capillary voltage was set to 4000V in positive ion mode, and to

4000V in negative ion mode. The nozzle voltage was 300V. The fragmentor voltage was 380V, the skimmer 45V and the OCT 1 RF Vpp 750V. The collision energy was set to 0V. The m/z range was 70-1700, and data was collected in centroid mode with an acquisition rate of 4 scans/s (1977 transients/spectrum).

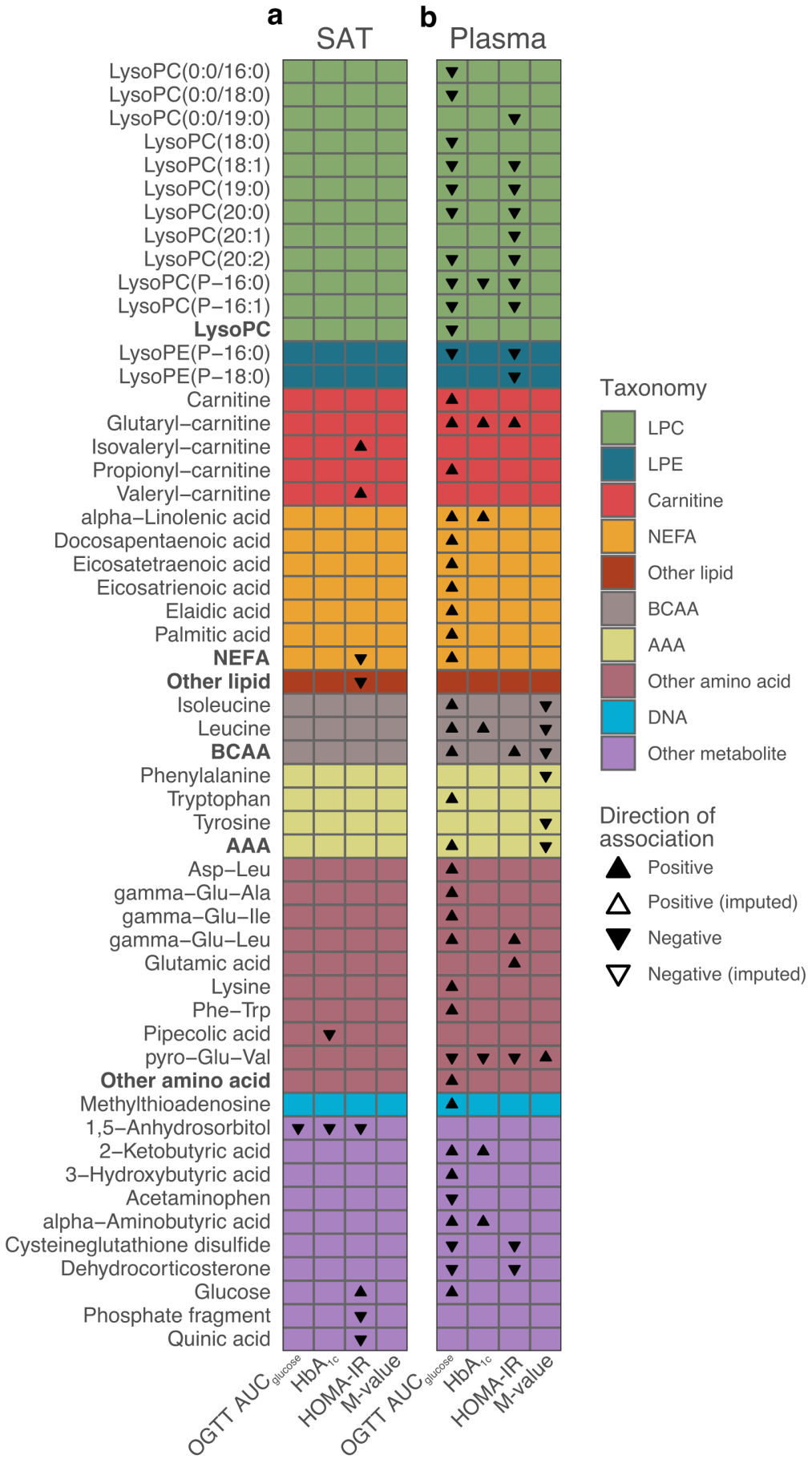
1.4 Metabolic profiling solvents

Methanol, HPLC-grade was obtained from Fischer Scientific (Waltham, MA, USA) Chloroform, Suprasolv for GC was obtained from Merck (Darmstadt, Germany) Acetonitrile, HPLC-grade was obtained from Fischer Scientific (Waltham, MA, USA) 2-Propanol, HPLC-grade was obtained from VWR (Radnor, PA, USA) H2O, Milli-Q. Reference and tuning standards: Purine, 4 μM, Agilent Technologies (Santa Clara, CA, USA) HP-0921 (Hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine), 1 μM, Agilent Technologies (Santa Clara, CA, USA) Calibrant, ESI-TOF, ESI-L Low Concentration Tuning Mix, Agilent Technologies (Santa Clara, CA, USA) HP-0321 (Hexamethoxyphosphazine), 0.1 mM, Agilent Technologies (Santa Clara, CA, USA). Stable isotopes internal standards: LC-MS internal standards: 13C9-Phenylalanine, 13C3-Caffeine, D4-Cholic acid, D8-Arachidonic Acid, 13C9-Caffeic Acid were obtained from Sigma (St. Louis, MO, USA). GC-MS internal standards: L-proline-13C5, alpha-ketoglutarate-13C4, myristic acid-13C3, cholesterol-D7 were obtained from Cil (Andover, MA, USA). Succinic acid-D4, salicylic acid-D6, L-glutamic acid-13C5,15N, putrescine-D4, hexadecanoic acid-13C4, D-glucose-13C6, D-sucrose-13C12 were obtained from Sigma (St. Louis, MO, USA).

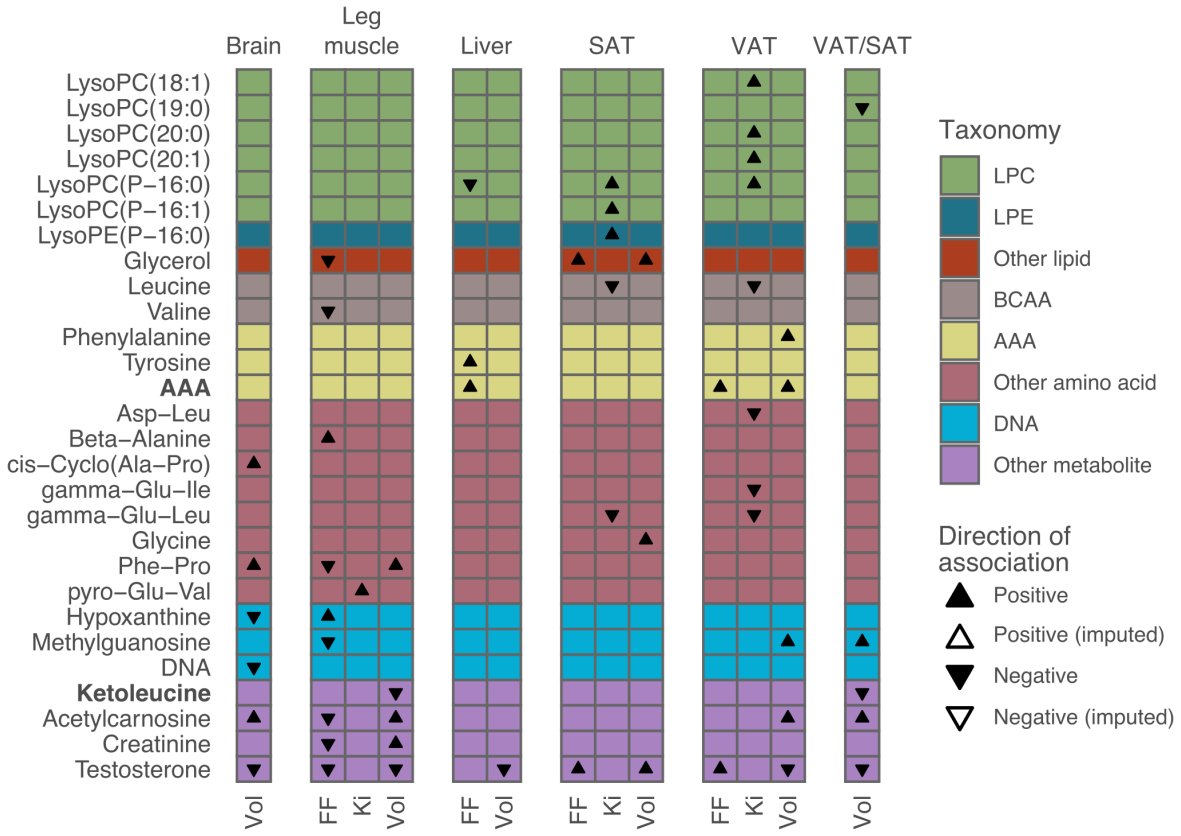
S3. Pathway and taxonomy enrichment analysis

We used the human metabolome database (HMDB)[4] identifiers of differentially significant metabolites ($p<0.1$) or metabolites significantly associated with M-value, OGTT $AUC_{glucose}$, HOMA-IR or HbA_{1c} for each tissue. We explored the taxonomy and pathway enrichment using MBROLE 2.0 (Supplementary Tables S5 and S6)[5]. We used the full set of metabolites for homo sapiens as background.

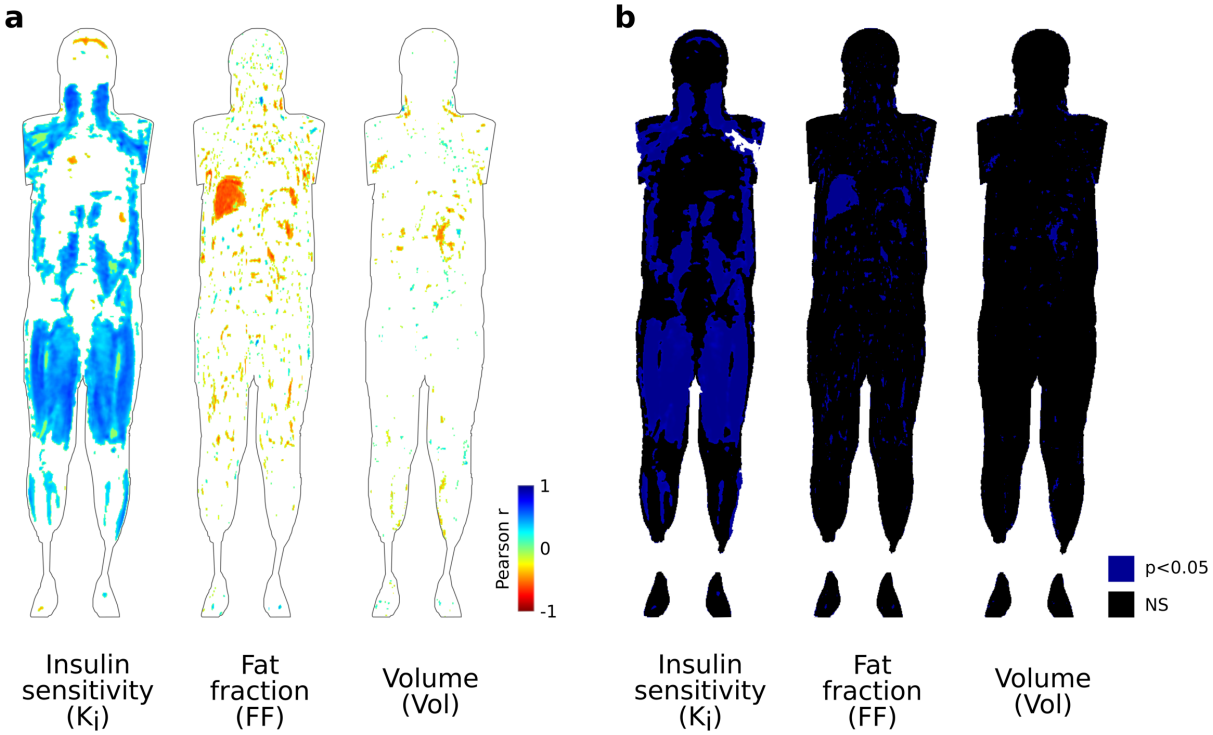
SUPPLEMENTARY FIGURES



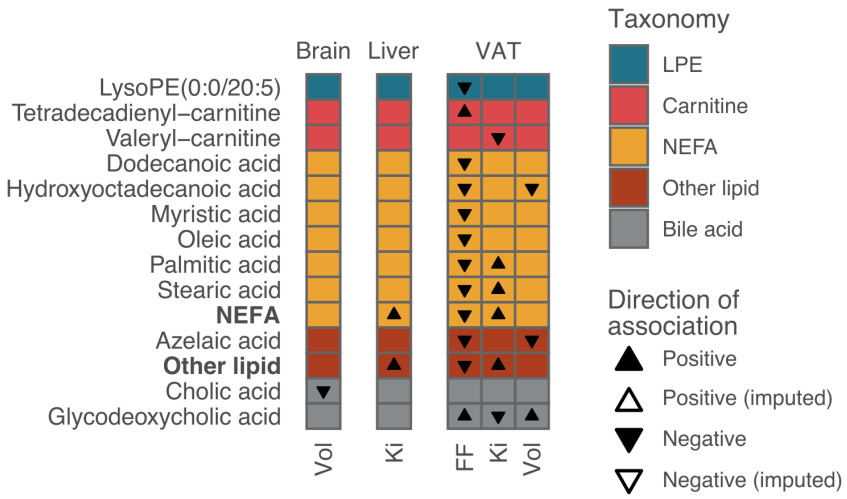
Supplementary Figure S1: Collection of metabolites associated with at least one of the T2D markers OGTT $AUC_{glucose}$, HbA_{1c}, HOMA-IR or M-value, in at least one tissue. Associations were calculated as described in methods. Classes of metabolites were identified on a manual curation of the HMDB annotations[4]. Direction of the association was decided based on the sign of the regression betas. Pools of metabolites are marked in bold. Significant associations originating from imputed metabolomics data points are marked as shown in the legend.



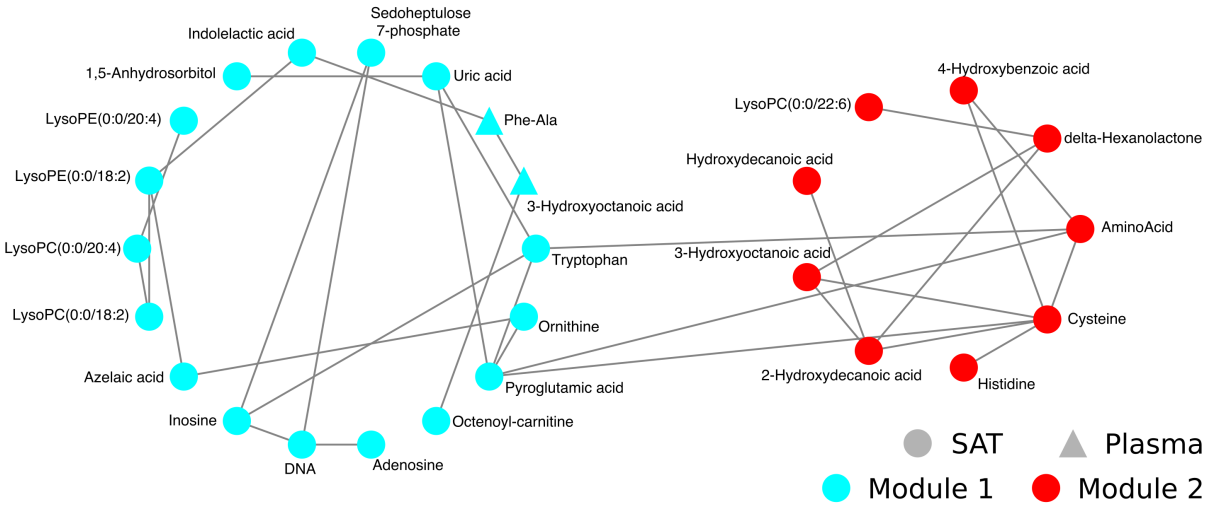
Supplementary Figure S2: Collection of metabolites associated with PET/MRI measurements in plasma. Associations were calculated as described in methods. Classes of metabolites were identified on a manual curation of the HMDB annotations[4]. Direction of the association was decided based on the sign of the regression betas. Pools of metabolites are marked in bold. Significant associations originating from imputed metabolomics data points are marked as shown in the legend.



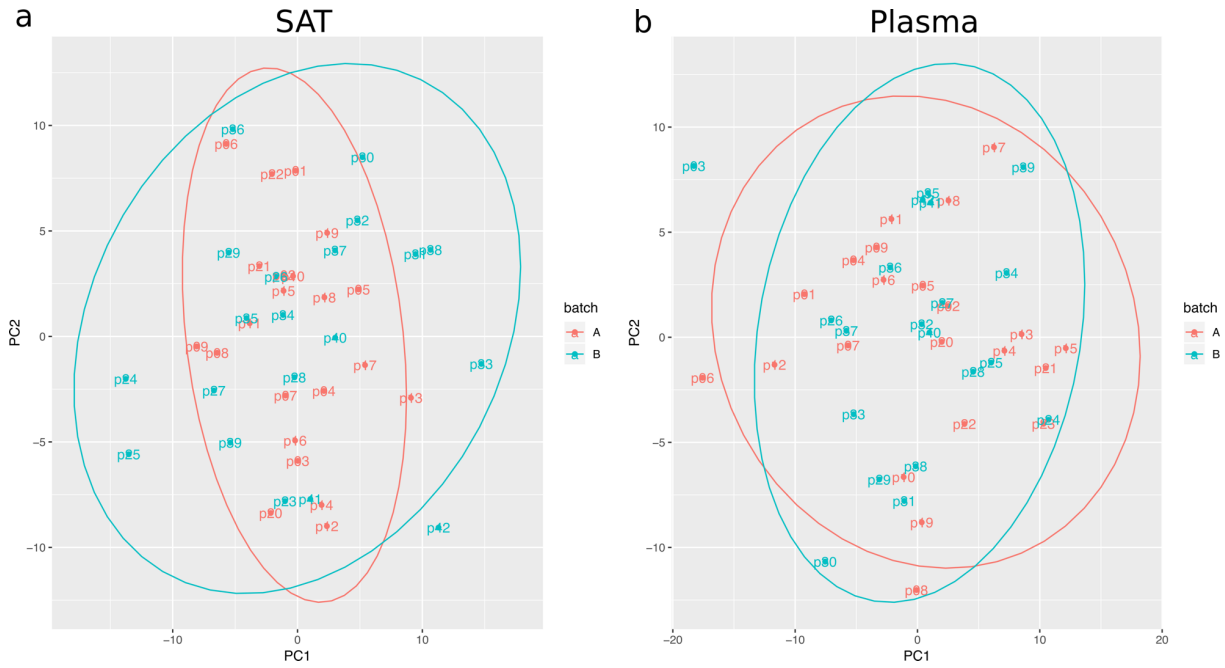
Supplementary Figure S3: Voxel-level correlation maps between lysoPC(P-16:0) and tissue parameters generated with Imiomics corrected for BMI, WHR, sex and age. **a)** Pearson's r-coefficient maps showing only significant associations. **b)** P-value maps thresholded at $p<0.05$ and converted to masks, displaying only significant voxel-level associations.



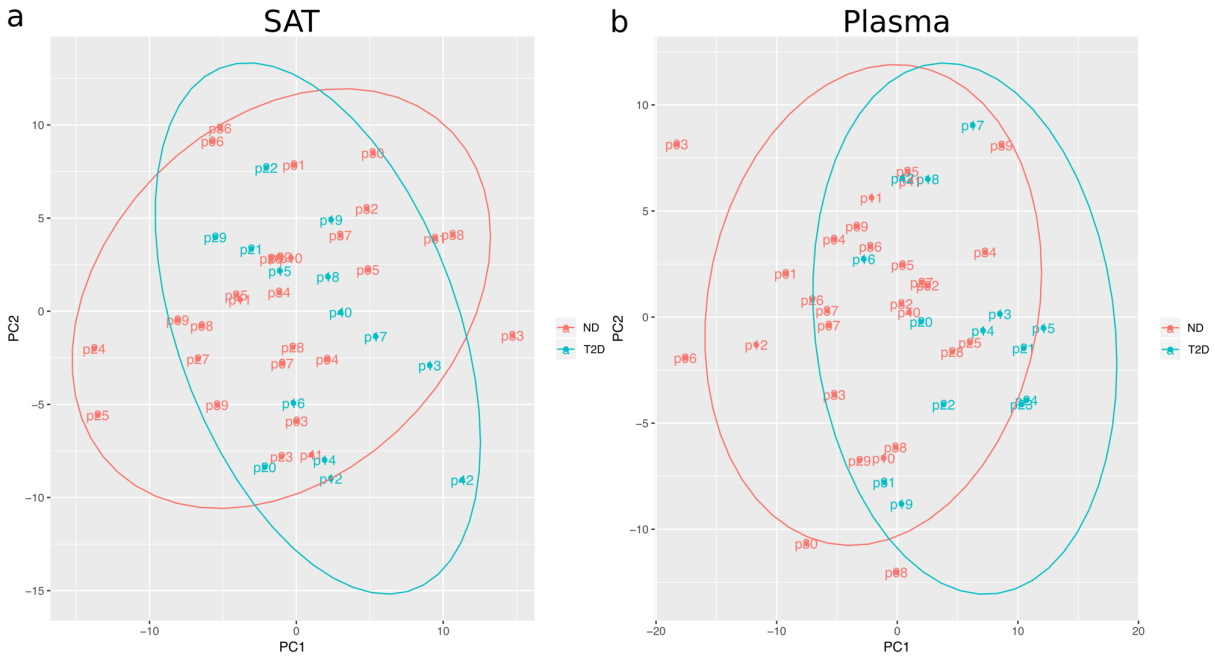
Supplementary Figure S4: Collection of metabolites associated with PET/MRI measurements in SAT. Associations were calculated as described in methods. Classes of metabolites were identified on a manual curation of the HMDB annotations[4]. Direction of the association was decided based on the sign of the regression betas. Pools of metabolites are marked in bold. Significant associations originating from imputed metabolomics data points are marked as shown in the legend.



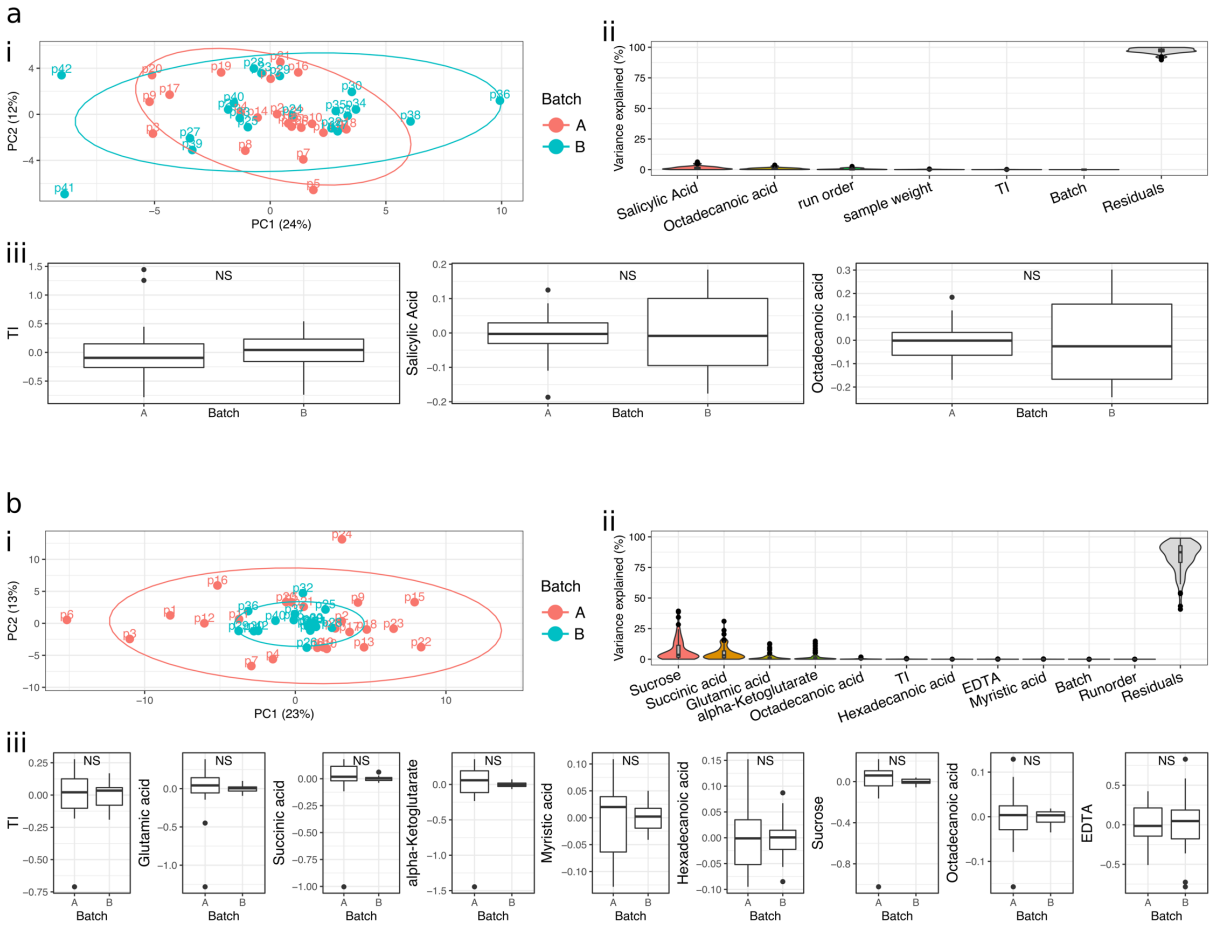
Supplementary Figure S5: Modules of metabolites detected by MoDentify as significantly associated with whole body insulin sensitivity (M-value)[1]. Nodes represent metabolites detected in SAT or plasma. Shape of node marks the tissue of origin of the metabolite. Color marks modules. Edges represent significant partial correlations of pairs of metabolites.



Supplementary Figure S6: PCA plots showing the two first principal components colored by batches on LC- and GC-MS metabolomics data after correction for MS run-order, internal standards, total intensity and batch in **a)** SAT, and **b)** plasma.



Supplementary Figure S7: PCA plots showing the two first principal components colored by binary phenotypic classes ND or T2D on LC- and GC-MS metabolomics data in **a)** SAT, and **b)** plasma.



Supplementary Figure S8: Quality control plots to explore batch effect due to the differing GC-MS platforms. **a)** and **b)** represent a collection of plots that refer to SAT and plasma, respectively. *i)* PCA plot for the first two principal components for GC-MS measurements colored by batch. *ii)* Violin plot illustrating the percentage of variance of all the metabolites explained by MS confounding factors including TI, IS, batch and MS running order. Values were computed using the R library variancePartition that calculates the percentage of variance of each metabolite explained by various confounding factors. *iii)* A collection of boxplots illustrating the levels of TI and IS in each batch. Statistical significance in each boxplot was computed from an unpaired two-sided Wilcoxon rank sum test. NS stands for no significance.

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